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THE ORIGIN, DISTRIBUTION AND FATE OF THE MOLTING FLUID PROTEINS OF THE CECROPIA SILKWORM

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Molting in insects is accompanied by the production of a molting fluid which fills the exuvial space between the new and old cuticles and digests the inner layers of the old cuticle. Most of the products of digestion are resorbed. This process has been studied in detail in the Cecropia silkworm by Passonneau and Williams (1953). During the pupal-adult transformation of the Cecropia silkworm, molting fluid appears in the exuvial space as soon as the epidermis retracts from the old pupal cuticle. This fluid persists for the first 19 days of the 21 days of pharate adult life. For the first two-thirds of this period the molting fluid is gel-like and has no obvious effect on the overlying pupal cuticle. However, on about the fourteenth day of pharate adult development, the molting gel of the pharate adult liquifies and begins to hydrolyze the proteins and chitin of the pupal endocuticle. By the 20th day the endocuticle has disappeared, leaving a thin crisp exocuticle and epicuticle. On the 19th day, molting fluid begins to be absorbed and, by the 20th day, most of it is gone from the exuvial space. On the 21st day, the insect emerges.

It is generally believed that the epidermal cells secrete the molting fluid. Whether they synthesize all of it, or secrete into it some components that were synthesized elsewhere, is not known. The literature contains only scanty references to the protein components of molting fluid. Passonneau and Williams (1953) showed that the early molting gel of Cecropia contains about 5 per cent protein. They also demonstrated proteolytic and chitinolytic activity in the molting fluid. Chen and Levenbook (1966) examined the molting fluid and blood of the blowfly, *Phormia regina*, by disc electrophoresis and reported that both fluids contained several proteins with similar R_f values. Their electrophoretic techniques did not permit them to determine whether any proteins of the molting fluid were related to those of the blood, or were products of cuticular digestion.

The mechanism of absorption of molting fluid prior to ecdysis is also unknown. It is generally believed that the ability to absorb molting fluid is a generalized property of the integument and that absorption takes place through the *general surface* of the new cuticle (Passonneau and Williams, 1953; Wigglesworth, 1933, 1948, 1965). However, it is not known whether specific regions of the integument are specialized as sites of absorption. Also, although it has been shown that small

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molecules are absorbed (*cf.* review by Noble-Nesbitt, 1967), nothing is known of the possible absorption of macromolecules.

This report examines the proteins of molting fluid, epidermis and blood during the pupal-adult transformation of the Cecropia silkworm, by immunological and electrophoretic techniques. It considers the following questions:

1. Which proteins (if any) are found in both molting fluid and blood, and where are these proteins made?
2. To what degree do native proteins (from blood and molting fluid) and introduced foreign proteins (from rabbit serum) move from molting fluid to blood and vice versa during adult development. How much macromolecular "traffic" is there between the blood and the fluid in the exuvial space?
3. Are macromolecules absorbed when molting fluid is absorbed just prior to ecdysis?
4. Is molting fluid absorbed by the general surface of the newly formed integument or does absorption occur more readily at certain sites?

Materials and Methods

Experimental animals

Larvae, pupae and pharate adults of the Cecropia silkworm, *Hyalophora cecropia*, were used in most experiments. Males and females were used interchangeably since there appeared to be no sex-limited differences in the phenomena we examined. In a few experimnts larvae of *Samia cynthia ricini* and *Galleria mellonella* were used. Methods of rearing, storing, handling and staging these insects have been described elsewhere (Krishnakumaran, Berry, Oberlander and Schneiderman, 1967; Schneiderman and Williams, 1954; Telfer and Rutberg, 1960). All animals were maintained at 25° C.

Reagents

Chemicals used for disc electrophoresis and immunoelectrophoresis were routine commercial preparations. Serum proteins came from laboratory rabbits. Purified rabbit gamma globulins (7S and 19S) were obtained by fractionation on DEAE cellulose columns and were lyophilized. All of the insect tissues were washed in 0.85% NaCl.

Collection of fluid in tissue samples from developing adults

Molting fluid, blood and tissue samples were taken from Cecropia silkworms of specific ages. Phenylthiourea (PTU) was added to all wounds and to all samples of tissue and fluids. In most experiments the fluids or tissue homogenates were centrifuged at 15,000 rpm in a Beckman "microfuge" for four minutes and stored at −20° C until used.

Molting fluid: A small piece of cuticle was excised from the tip of the abdomen, and approximately 200 μl of molting fluid was collected in a capillary. During this process it was necessary to avoid touching the delicate epidermis with the capillary. Otherwise, tissue damage occurred and caused blood and molting fluid

to mix. Molting fluid collected in the way described was not contaminated by blood (see Results). Occasionally, molting fluid was collected from antennae or wings for special purposes, but this fluid was often contaminated by blood.

Blood: Blood was collected from larvae, diapausing pupae and adults by means of fine glass needles inserted into the heart. To avoid contaminating the blood of pharate adults with molting fluid, special procedures were employed. In early pharate adults, an incision was made through the pupal cuticle along the dorsal midline of the abdomen. At this time, the epidermis is still closely pressed against the cuticle above the heart, and blood was collected from the heart without contamination by molting fluid. In late pharate adults, the pupal cuticle was removed, the animal washed in saline and blotted to avoid contamination by molting fluid. A sharp capillary was then inserted through the cuticle of the pharate adult into the heart to collect blood.

Epidermis: The developing wings and antennae are a convenient source of epidermis. However, the blood present in these appendages made it impossible for us to obtain epidermis free of blood, even though the tissues were cut into small fragments and washed repeatedly. In contrast, the abdominal epidermis could be isolated and freed of blood by careful dissection, fragmentation and repeated washing in ice cold saline. The fragments were blotted to remove excess saline, homogenized, centrifuged and the clear supernatant used for further analysis. Abdominal epidermis prepared in this way had no detectable amounts of major blood proteins.

Disc electrophoresis

For disc electrophoresis the procedure of Davis (1964) was followed, using *tris*-glycine buffer (pH 8.6) and applying 3 MA per gel for 30 minutes at 25° C. The procedure was modified slightly to meet our special needs (*cf.* Patel and Schneiderman, 1969 for details). Gels of several pore sizes were tested. A 4.3% gel provided the best resolution for blood, molting fluid and epidermal proteins and was employed in most experiments. Gels were stained after electrophoresis with Buffalo Black (=Naphthol Blue Black, Allied Chemicals) and were destained and preserved in 10% acetic acid.

Immunological methods

Antisera to blood proteins of diapausing pupae and adults of male and female Cecropia silkworms were prepared in rabbits. The insect blood was emulsified in Freund's adjuvant and injected subcutaneously into rabbits in four to six sites along the vertebral column. Two weeks later boosters were injected into the same sites. The antisera were deepfrozen at −20° C until used. These antisera could detect up to twelve different Cecropia blood antigens in immunoelectrophoresis experiments. Sheep antisera against total rabbit serum proteins and against rabbit gamma globulins were prepared by conventional methods.

The immunoelectrophoretic procedures of Grabar and Williams (1955) and the modified technique of Scheidegger (1955) were used to detect blood antigens in various fluids and tissues. Standard microscope slides were covered with 2 ml of

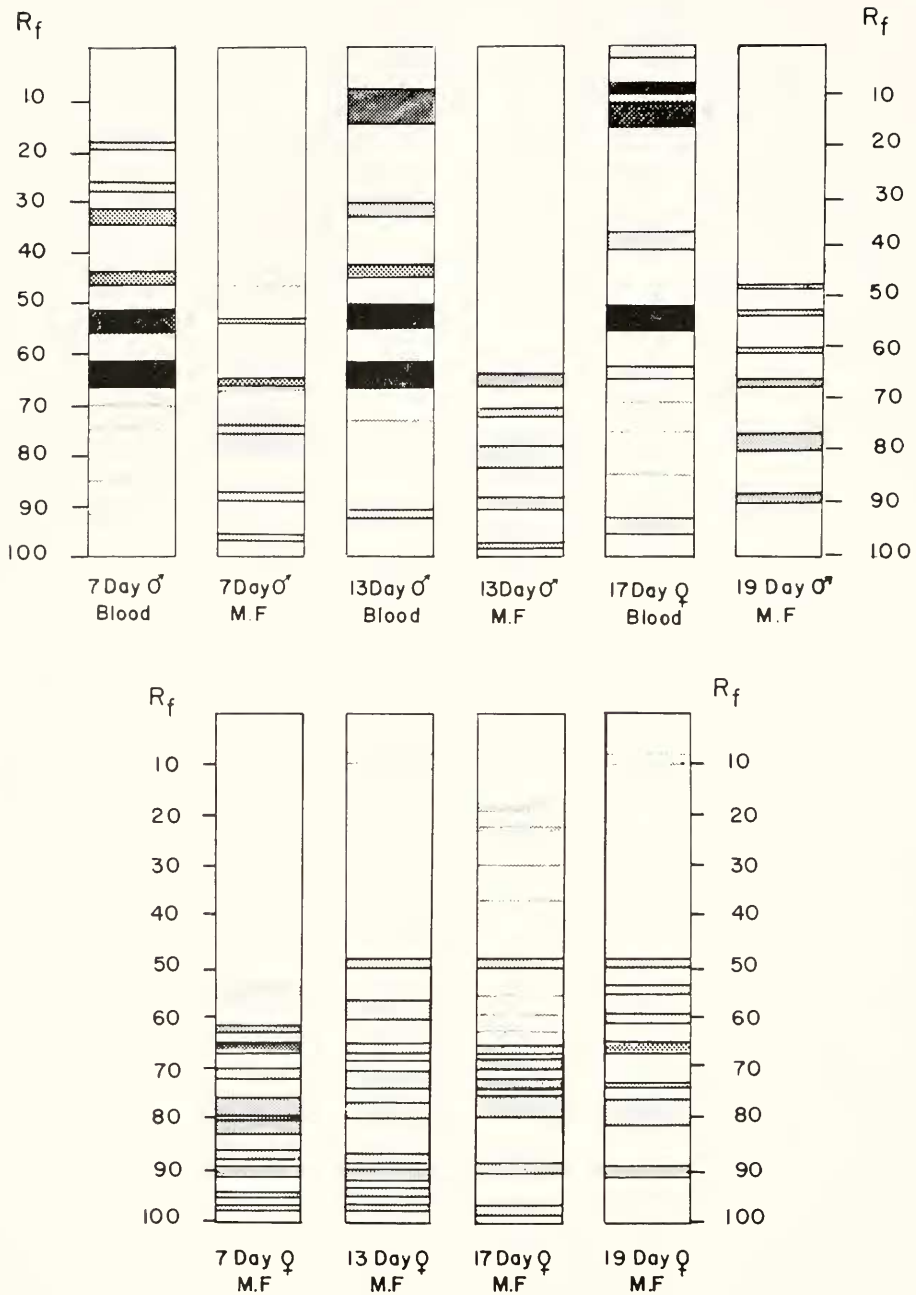

FIGURE 1. Typical acrylamide gel disc electrophorograms of blood and molting fluid proteins of Cecropia moths at various stages of adult development. In all cases 2 μl of blood or 10 μl of molting fluid were applied. Each drawing represents an actual gel. All samples were mixed with an equal volume of upper gel solution saturated with sucrose before photopolymerization.

1% agar (Difco, Special "Noble") in barbital acetate buffer at pH 8.6, $\mu = 0.025$. Troughs and holes were cut in agar as follows: 3 holes (1 mm diameter, 5 mm apart) and two troughs (6 mm apart) (Figs. 3 and 5). Electrophoresis was performed at 200 volts, applying 7 to 8 MA per slide for 60 minutes at 3° C. After electrophoresis, the separated proteins were reacted with rabbit sera against the blood of Cecropia pupae and adults. Precipitation arcs appeared after 12 to 48 hours at 25°, slides were washed in saline for three days, dried and stained with Buffalo Black.

Ouchterlony's double-diffusion analysis (Ouchterlony, 1958) was carried out in 5 cm Petri dishes covered with 4 ml of 1% agar in barbital acetate buffer. One central and 6 circumferential wells were cut out with a 13 gauge syringe needle and spaced 5 mm apart from each other (Fig. 6). Diffusion took place for 12 to 48 hours at 25° C.

Injection of dyes and proteins into the exuvial space and body cavity

Rabbit serum and purified rabbit gamma globulins were used as foreign proteins to help us detect macromolecular "traffic" between the exuvial space and the body cavity. Their presence in fluid compartments and in tissues was tested for by double diffusion analysis using sheep antiserum against rabbit gamma globulin. The distribution and penetration of macromolecules from one fluid compartment into another were also studied, using both rabbit serum proteins and molting fluid proteins stained with Buffalo Black. A series of dialysis studies with rabbit serum proteins stained with Buffalo Black demonstrated that, at concentrations of 0.1%, the dye remained firmly bound to the protein for at least 24 hours. This staining technique provided us with an easy way of tagging various proteins and studying their movements between fluid compartments. It was always confirmed by immunological methods.

Injection of foreign proteins, dye-"tagged" proteins or dye alone was made either into the exuvial space or into the body cavity in the dorsal side of the second or third abdominal segment.

Injections into the exuvial space posed no problem. However, special techniques were required to prevent blood and molting fluid from mixing during injections into the body cavity. In pharate adults younger than 14 days, the injection was made in the second or third abdominal segment through the pupal cuticle and directly into the heart. The epidermis in this region is closely pressed against the cuticle and no detectable mixing of blood and molting fluid occurred. In older pharate adults, the following procedure was carried out to prevent the mixing of blood and molting fluid during injection or immediately thereafter. A 6 mm square of pupal cuticle was excised between the second and third abdominal segment above the heart and the exposed edges of the pupal cuticle were sealed to the adult cuticle with melted paraffin. The small depression formed by this procedure was washed with saline and a volume of blood comparable to the amount of solution to be injected was removed from the heart. The experimental solution was introduced into the heart, and the exposed area sealed with paraffin.

Results

Compartmentalization of the exuvial space

The first experiments were designed to determine whether compartmentalization exists within the exuvial space. About half of the molting fluid of the left antennal case of a 16-day old pharate adult was removed through an opening in the pupal cuticle and replaced by a mixture of molting fluid tagged with Buffalo Black. One day later, examination revealed that the stained protein had not diffused from the antennal case. In a second experiment, a similar injection was made into the left antennal case of a 20-day old pharate adult. Five hours after the injection, the Buffalo Black-tagged protein had spread from the left antennal case to the fore and hind wings, head and thorax.

In another experiment on a 20-day old pharate adult 1 ml of 1% aqueous solution of Buffalo Black was injected into the exuvial space at the tip of the abdomen. Ten hours after the injection, the Buffalo Black-tagged protein had diffused into the wings, thorax and head.

These experiments were repeated several times with the same results. Clearly, the exuvial space is compartmentalized and the fluids in the compartments do not mix with one another until several days before emergence. For this reason, all samples of molting fluid used in this study were withdrawn from the same place, namely the tip of the abdomen. Apparently, molting fluid is formed separately in several regions between which parts of the pupal cuticle remain attached to the epidermis. These areas of persistent attachment effectively divide the exuvial space into compartments. Just prior to ecdysis, the pupal cuticle detaches in these areas, and the exuvial space becomes continuous.

The protein composition of blood and molting fluid as revealed by disc electrophoresis

Samples of blood and of molting fluid were taken from pharate adults at various stages of the pupal-adult transformation and from diapausing pupae. The samples were subjected to disc electrophoresis and some typical results are recorded in Figure 1. From an analysis of the gels illustrated in this Figure and more than 50 similar gels, the following facts emerged:

(1) The molting fluid was not contaminated by blood, because it was colorless. The slightest contamination could be detected by the occurrence of yellow pigments. Also, as Figure 1 shows, several of the densest blood protein bands (R_f 34 and 45) were absent from molting fluid.

(2) None of the major protein bands in the blood was a major component of the molting fluid and vice versa. Band R_f 66, a major blood protein component, was detectable in low concentrations in the molting fluid. All 5 major blood protein bands had R_f's between 0 and 66 while major molting fluid protein bands had R_f's greater than 66.

(3) Molting fluid contained at least 10 proteins which were not detected in the blood.

(4) Profound changes occurred in the protein pattern of the blood and molting fluid during development. Only changes that occurred in the molting fluid will

be discussed here. The molting fluid on day 7 is of special interest because it is still inactive enzymatically and does not contain products of cuticular digestion. It contained 8 to 10 distinct protein bands, but none had R_f's less than 45. However, between day 13 and 17, a number of new bands appeared with R_f's ranging from 10 to 38, some of which persisted throughout development. Whether these proteins have anything to do with the enzymatic activity of the molting fluid, or include products of cuticular digestion, will be considered in the discussion.

(5) A number of bands in blood and molting fluid have similar R_fs. Which of these bands represented identical proteins is considered in the next section.

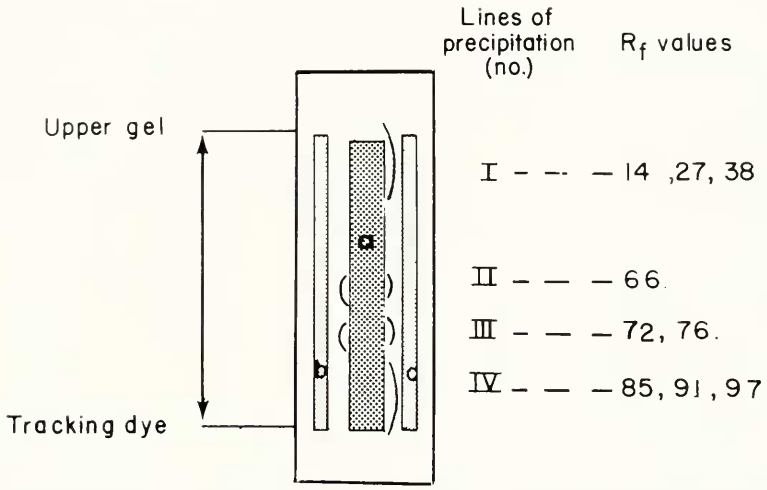

FIGURE 2. Diagram showing results of combined disc electrophoresis and immunodiffusion of molting fluid of 17-day old developing adult male Cecropia moth. The results reveal four lines of precipitation (I–IV) indicating the presence of four blood antigens in the molting fluid. The R_f values of the molting fluid protein bands which correspond to the lines of precipitation are indicated. It was not possible to determine which of several protein bands corresponded to each line of precipitation. Stipples indicate (a) unfixed half of acrylamide gel; (b) rabbit antiserum against blood of male pupae; and (c) rabbit antiserum against blood of male adults.

Proteins common to molting fluid and blood

The following electrophoretic and immunological methods were used to determine which protein bands with similar R_fs in molting fluid and blood are identical.

Separation by disc electrophoresis and precipitation by antisera against blood proteins: Thirty μl of molting fluid from 17-day old pharate adult males were applied to each acrylamide gel and the proteins separated as previously described. Following electrophoresis, the gels were cut longitudinally into two halves; one was stained with Buffalo Black to locate the protein bands and the other half was placed on a microscope slide coated with 2% agar. Rabbit antisera against male pupal blood and against male adult blood were added into two parallel troughs on each side of the split gel. Diffusion was allowed to occur until no new lines of

precipitation appeared. The results are recorded in Figure 2. Four lines of precipitation were formed with antiserum against adult blood serum and two with antiserum against pupal blood. The R_f values of the molting fluid protein bands which could correspond to each of these lines are recorded in Figure 2. It is impossible to decide precisely which of these bands are the blood antigens.

From this result it is clear that the molting fluid of 17-day old pharate adults contains 4 antigens found in adult blood. Two of these antigens are absent from pupal blood or present in much lower concentrations. Further support for these conclusions comes from immunoelectrophoretic data.

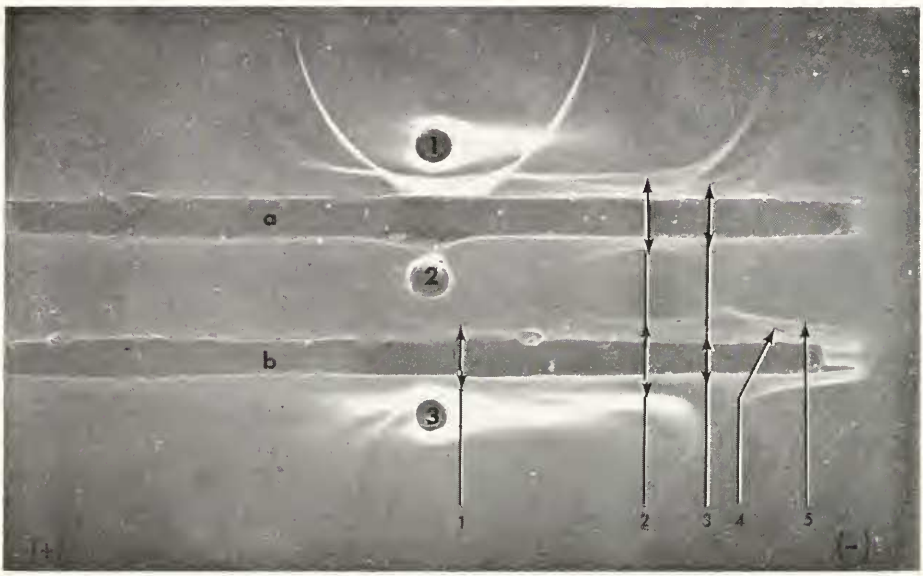

FIGURE 3. Immunoelectrophoretic analysis of blood and molting fluid of 16-day old developing adult male Cecropia moth. The wells contained (1) 2 μl of blood, (2) 10 μl of molting fluid, and (3) 2 μl of blood. The longitudinal troughs had (a) rabbit antiserum against blood of male pupae, and (b) rabbit antiserum against blood of male adults. The numbered arrows indicate the lines of precipitation formed by blood antigens present in the Cecropia blood and molting fluid.

Immunoelectrophoresis: Molting fluid and blood from pharate adults 13, 16, 19 and 20 days old were analyzed by precipitation with rabbit antisera against the blood of male pupae and against the blood of female pupae. Figure 3 shows a typical result obtained with a 16-day old pharate adult. The many lines formed by the blood need not concern us. The key result is the demonstration that molting fluid formed two lines of precipitation with antiserum against pupal blood and five lines with antiserum against adult blood. These lines corresponded to those formed by blood antigens as follows:

(1) Lines 2 and 3 formed with antiserum against male pupal blood. A diffuse line, No. 1, also formed but is not visible in the photograph.

(2) Lines 1 to 5 formed with antiserum against male adult blood. There was

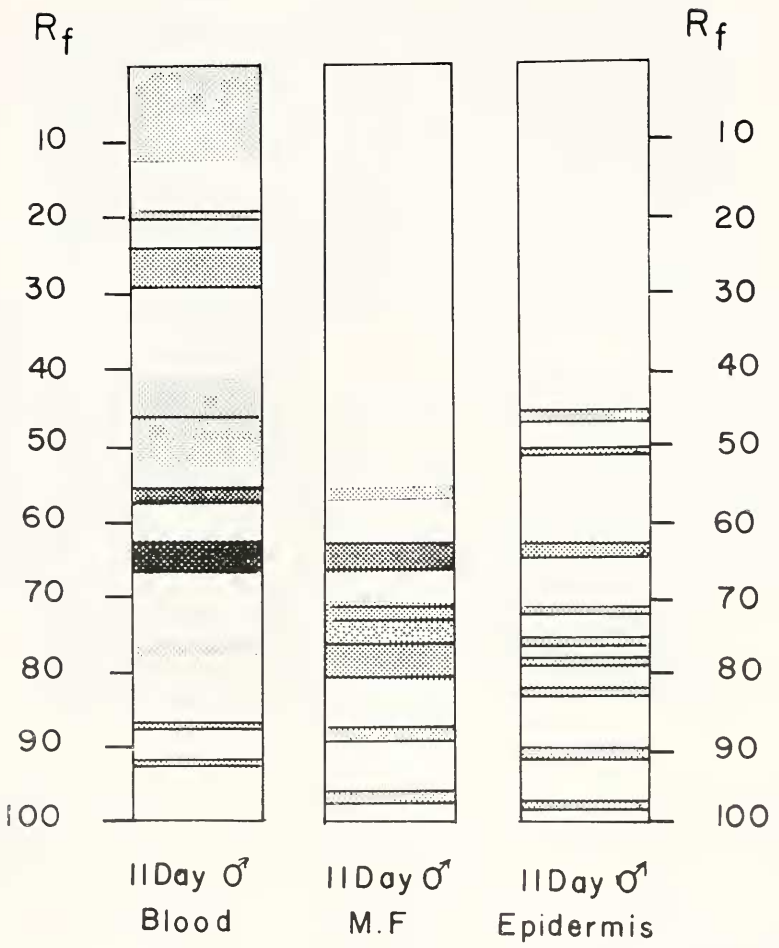

FIGURE 4. Acrylamide gel disc electropherograms of blood, molting fluid and epidermis of an 11-day old, pharate, adult male Cecropia moth.

no basic change in the number or identity of the blood antigens present in molting fluid during the final seven days of pharate adult development. These results confirm and extend the combined disc electrophoresis-double diffusion experiments described above.

Ouchterlony double diffusion analysis confirmed the identity between several blood antigens and molting fluid antigens (*cf.* also results presented in section 4(c) below and Figure 6).

The origin of proteins common to blood and molting fluid

It is generally believed that the epidermis is "the source of at least the principle constituents of the molting fluid" (Passonneau and Williams, 1953). To deter-

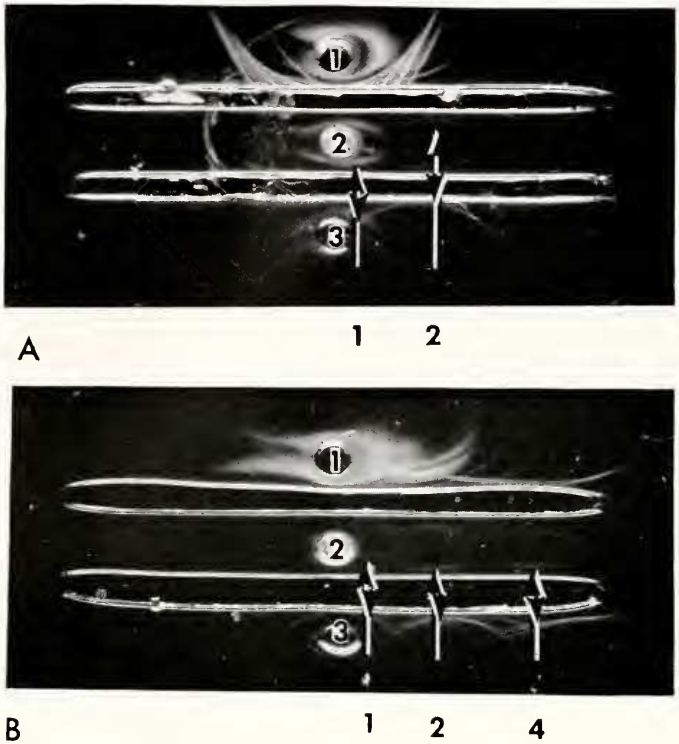

FIGURE 5. Immunoelectrophoretic analysis of blood, molting fluid and epidermis of 11-day old, pharate, adult male Cecropia moth. The wells contained (1) 2 μl of blood, (2) 10 μl of epidermal homogenate, and (3) 10 μl of molting fluid. In (A) rabbit antiserum against blood of male pupae was in both troughs, and in (B) rabbit antiserum against blood of male adults in both troughs. The numbered arrows indicate the lines of precipitation formed by Cecropia blood antigens. Lines 3 and 5 did not show up in the photograph.

mine whether the epidermis was the source of the five antigens found in both the molting fluid and blood, epidermal proteins were examined by disc electrophoresis, immunoelectrophoresis and double diffusion.

Figure 4 records the results of disc electrophoresis of epidermis, blood and molting fluid from 11-day old pharate adults and demonstrates that the epidermis and molting fluid have a number of proteins with the same R_f values. They both lack the same major blood proteins. In short, in terms of disc electrophoresis patterns, epidermis resembles molting fluid more than blood.

The identity between certain epidermal, molting fluid and blood proteins was established by the following immunoelectrophoretic analyses. Blood, molting fluid and epidermis from 11-day old pharate adults were subjected to immunoelectrophoresis using antisera against male pupal blood and male adult blood. It is evident from the results in Figure 5 that blood antigens 1, 2 and 3 were present both in the epidermis and the molting fluid when reacted with antiserum against pupal blood. Lines of precipitation 1, 2, 4 and 5 were formed by the epidermis

and lines 1 to 5 by the molting fluid following their reaction with antiserum against adult blood. The yellow pigments characteristic of both pupal and adult blood, which are bound to certain blood proteins, were absent from the abdominal epidermis as well as from the molting fluid of pharate adults.

These observations show that epidermis contains at least 4 of the 5 blood antigens found in the molting fluid. Proof of the identity among the antigens in the epidermis, blood and molting fluid was established by Ouchterlony double-diffusion analysis as follows: Molting fluid, blood and abdominal epidermis of 11-day old pharate male adults were examined by the Ouchterlony double-diffusion technique

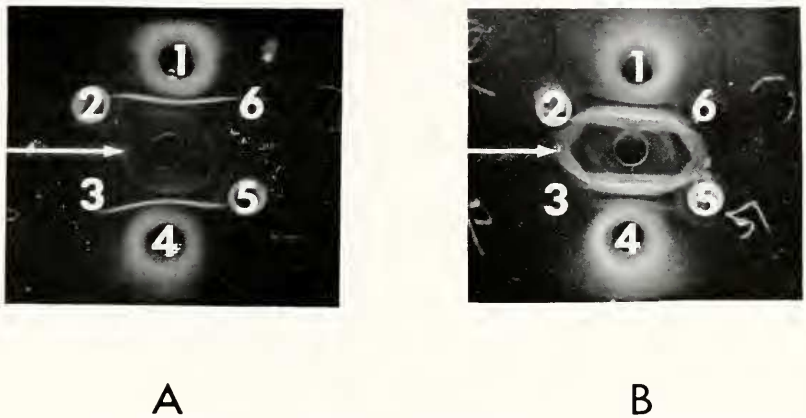

FIGURE 6. Double diffusion analysis of blood, epidermis and molting fluid of 11-day old, pharate, adult moth absorbed with two different antisera. Surrounding wells were prepared with (1, 4) blood, (2, 5) abdominal epidermis, and (3, 6) molting fluid. In (A) the center well contains rabbit antiserum against male pupal blood, and in (B) the center well contains rabbit antiserum against male adult blood. Blood antigens present in these three protein mixtures which fuse and form lines of identity are indicated by arrows. Another line of identity in (A) between wells 2 and 3 and between wells 5 and 6 did not show up in the photograph.

for the presence of lines of identity between these three protein mixtures. Rabbit antisera against male pupal blood and male adult blood were used. The results in Figure 6 reveal that two identical lines of precipitation were formed by the molting fluid, epidermis and blood when tested with antiserum against pupal blood. Figure 6 also shows that at least two identical lines were formed by blood, molting fluid and epidermis when reacted with antiserum against adult blood. In both cases heavy lines of precipitation were formed only by the blood, and were absent from the epidermis and molting fluid.

Although the resolution of the double-diffusion technique is much less than that of immunoelectrophoresis, these observations are consistent with the results of immunoelectrophoresis and establish that several of the blood antigens present in molting fluid are identical with those present in the epidermis.

The route of entry of blood antigens into molting fluid

The occurrence of blood antigens in molting fluid raises the question of how they get there. Are they secreted by the epidermal cells into the exuvial space and also

into the blood? Or, do they originate in some other tissue, to be transported by epidermal cells into the exuvial space? Indeed, this raises the more general question of what sort of commerce there is between the molting fluid and blood compartments of the insect.

To answer this question, rabbit whole serum and rabbit whole serum tagged with Buffalo Black was injected into the abdominal exuvial space or into the body cavity of pharate adult females of different ages. Double diffusion analysis with sheep antiserum against rabbit gamma globulin was used to detect the presence of the gamma globulin fraction of the injected serum in various fluid compartments and tissues. The distribution of Buffalo Black-tagged proteins was visually determined. Unless otherwise indicated each experiment was repeated on three animals.

Injection of rabbit serum proteins into the exuvial space: In the first experiment 200 μl of molting fluid was removed from 7-day old pharate adults. Following this, 150 μl of rabbit serum was injected into the exuvial space and the opening sealed with paraffin. Four or ten days after the injection, the animals were sacrificed and blood and molting fluid were analyzed by double diffusion. The results revealed that rabbit gamma globulins were detected in the molting fluid but not in the blood. Apparently, in 11- to 17-day old pharate adults, the rabbit serum proteins did not move from the exuvial space into the blood. Also, the rabbit serum proteins retained their antigenic activity for at least ten days in the molting fluid, notwithstanding the presence of proteolytic enzymes in this fluid (Passonneau and Williams, 1953).

In another experiment, between 20 and 100 μl of whole rabbit serum protein were injected into the exuvial space of 19-day old pharate adults at the peak of molting fluid absorption. Twenty-four hours later, blood and molting fluid were tested by double diffusion analysis for the presence of rabbit serum proteins using sheep antiserum against whole rabbit proteins and sheep antiserum against rabbit gamma globulin. No rabbit serum proteins were detected in the blood, but they were found in the molting fluid.

In a third experiment 250 μl of whole rabbit serum was injected into the exuvial space of a 20-day old pharate adult. The insect resorbed its molting fluid four days later, whereupon blood, fat body and ovaries were examined by double diffusion analysis. No lines of precipitation were observed. Apparently, immunologically active rabbit serum proteins are not resorbed into the blood even when active molting fluid absorption takes place.

To summarize, foreign proteins injected into the exuvial space of pharate adults of different ages could not be detected in the blood of these animals by immunological methods. This finding indicates that macromolecules similar to rabbit gamma globulins do not penetrate the new adult cuticle and epidermis to enter the blood, prior to or during active molting fluid absorption.

Injection of rabbit serum proteins into the body cavity: The next question asked was whether macromolecules could cross the epidermis from the body cavity into the exuvial space. To answer this question, rabbit serum proteins were injected into the heart of pharate adults of different ages.

In the first experiment, 7-day old pharate adults were injected with 100 μl of rabbit serum and killed 4, 10 or 15 days thereafter. Double diffusion analysis

of molting fluid, fat body and blood revealed rabbit gamma globulins only in the blood. Thus, although rabbit globulins were detected in the blood for 15 days after injection, these proteins failed to pass from the blood into the exuvial space at any stage.

A similar experiment was performed on 14-day old pharate adults in which 350 μl of rabbit serum protein tagged with Buffalo Black were injected. The pharate adults were killed five days after the injection, and blood, molting fluid and various tissue were examined visually for the presence of blue dye, and immunologically by double diffusion, for the presence of rabbit serum proteins. The results revealed that the oocytes were the only tissue, besides blood, which contained gamma globulins. The oldest oocytes did not stain blue, but younger ones did, and the oocytes as a whole contained gamma globulins. However, when gamma globulins were injected on days 19 or 20, none of the oocytes took up gamma globulins. Although the Malpighian tubules were stained blue, no antigenically active gamma globulins were detected. Apparently the blue color is due to dye molecules which dissociated from rabbit serum proteins that might have been degraded.

Taken together, these experiments indicate that foreign proteins do not penetrate from the exuvial space to the blood or vice versa. The presence of certain native proteins in the blood and their absence from the molting fluid, coupled with the presence of certain native proteins in the molting fluid and their absence from the blood, also demonstrates an effective separation between the two fluid compartments. The results suggest that the blood antigens found in the molting fluid are not transported from the blood into the molting fluid, but are secreted by the epidermis directly into both molting fluid and blood.

Sites of molting fluid absorption

To identify the sites of molting fluid absorption, the fate of Buffalo Black-tagged molting fluid was investigated. In pharate adults, molting fluid is absorbed on days 19 to 21. A series of developing adults 17 to 18 days old had the pupal cuticle on the tip of the abdomen removed, 0.1 ml of molting fluid was collected and mixed with 0.9 ml of 2% Buffalo Black in saline. The resulting mixture was reinjected into the exuvial space. Four days later, after emergence, the adult integument was examined. Only one part of the abdominal integument showed conspicuous blue staining. This was a narrow band located on each intersegmental membrane close to the sclerotized ridge at the posterior margin of each segment (Fig. 7). This blue-staining band was made up of many small pits or depressions about 15 microns in diameter. These pits were the only part of the cuticular surface that stained blue (Fig. 8) and from which diffusion of blue dye down and laterally into the integument took place.

In another experiment pupal cuticle was removed from 19- and 20-day old pharate adults and a drop of Buffalo Black-tagged molting fluid was applied to various parts of the adult integument. The penetration of the blue dye through the pits could be followed under the microscope. Within 15 minutes the dye was absorbed through the pits and its centrifugal diffusion into the integument could be followed. It was absorbed only through the pits.

These pits absorbed Buffalo Black-tagged molting fluid only prior to ecdysis. If Buffalo Black-tagged molting fluid was applied to the integument after ecdysis, the dye-protein mixture accumulated *on* the pits, but could be washed away with saline. It failed to enter the integument, presumably, because of a waterproof coating on the pits.

Similar pits are also found in corresponding parts of larvae and pupae. They are permeable to Buffalo Black-tagged molting fluid only before ecdysis. In fifth-stage larvae of *Samia cynthia ricini*, for example, five to six hours after ecdysis, the pits failed to absorb Buffalo Black-tagged molting fluid. In pre-

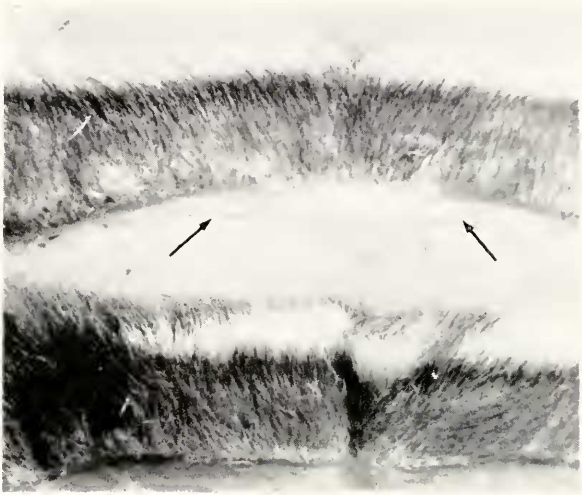

FIGURE 7. Portion of dorsum of abdomen of newly emerged female Cecropia moth showing tonofibrillar insertions in the intersegmental membrane (arrows). Buffalo Black dye was applied to the surface of the cuticle to emphasize the bands of pits for photographic purposes (× 16).

pupae of *Galleria mellonella* Buffalo Black-tagged molting fluid was also absorbed via pits, but after pupation there was no absorption.

These pits appear to be principal sites of molting fluid absorption in the abdomen. In the thorax, molting fluid is resorbed on the patagia, parapatagia, membranes at the bases of the legs and also in the membranes lying between epimeron 2 and 3 and subalare 2 and 3. In the head, some Buffalo Black-tagged molting fluid appeared to be sucked into the mouth and some was also absorbed by the integument beneath the mouth parts.

These pits mark the points at which tonofibrils occur. In the abdomen, the longitudinal muscles attaching adjacent segments and the lateral muscles within a segment make tonofibrillar insertions in these pits. In pharate adult Cecropia, the small portion of each tonofibril which attaches the pupal cuticle to the adult cuticle (and due to which the pharate adult is capable of moving the pupal cuticle) breaks down after about day 18. After this time, the absorption of molting fluid takes place through these pits in the adult integument.

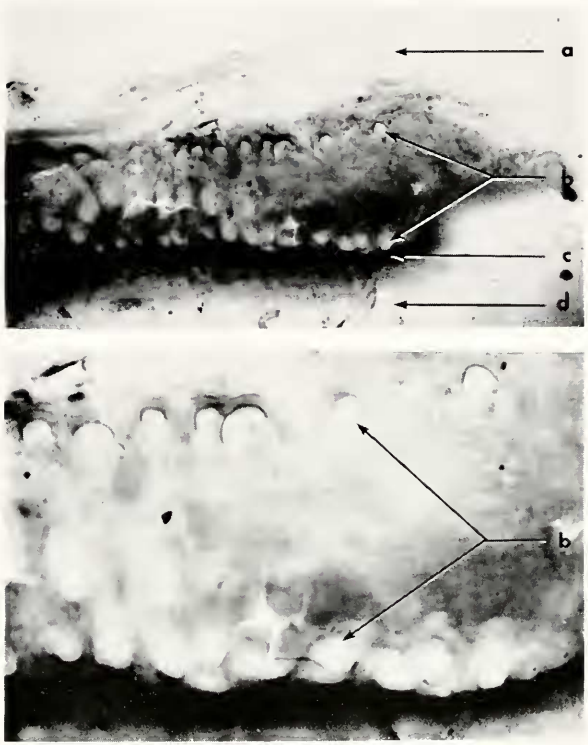

FIGURE 8. Tonofibrillar insertions showing absorption of stained molting fluid proteins in a portion of the intersegmental membrane of a 21-day old, pharate, male Cecropia moth. Approximately 250 μl of molting fluid tagged with Buffalo Black had been injected on day 18 into the exuvial space. Three days later, the animal was killed and a portion of the abdominal dorsum was free of adhering tissues and fixed. An overall view of a portion of a segment ($\times$ 320) is shown above (A), and a high power view of above ($\times$ 800) below (B). Labels indicate: (a) intersegmental membrane, (b) tonofibrillar insertions or "pits," (c) the sclerotized ridge, and (d) a segment showing scale sockets.

These experiments with Buffalo Black-tagged protein suggest that some macromolecules are absorbed from the molting fluid. Proof that this occurs was provided by the following immunological experiment. An injection of 275 μl of rabbit serum was made into the ecdysial space of an 18-day old developing adult. When it had emerged five days later the integument (adult cuticle plus epidermis) was freed of surrounding tissues and scales and washed thoroughly in saline. It was then homogenized and tested for the presence of rabbit serum proteins by double diffusion analysis. The results revealed rabbit serum proteins in the integument, while longitudinal muscles which are attached to the tonofibrils showed no reaction. Identical results were obtained with rabbit serum proteins tagged with Buffalo Black. These observations prove that the integument absorbs macromolecules along with molting fluid and these remain there for several days in an immunologically active state. The results indicate that in the normal process of absorption of molting fluid some macromolecules may be absorbed intact.

Principal sites of absorption in the abdomen are the tonofibrillar insertions. The absorbed proteins do not appear to enter the blood but some of them enter the integument.

Discussion

The results indicate that molting fluid is a separate fluid compartment and has at least 10 protein components which were not detected in the blood. These 10 components are not products of cuticular digestion since they are present in inactive molting fluid prior to cuticular digestion. The blood and molting fluid do share five antigens. One of these, a major blood protein band, is a minor component of molting fluid. The other four common protein components are minor compartments of both blood and molting fluid.

This different protein composition in the two fluid compartments depends on the epidermal cells which act as a barrier to most macromolecules in the molting fluid and the blood. The existence of this barrier is evident from the fact that numerous native blood proteins were never detected in the molting fluid, and many native molting fluid proteins were never detected in the blood. Moreover, Buffalo Black-tagged molting fluid proteins also failed to appear in the blood. Similar results were obtained with various rabbit serum proteins of different molecular weights which failed to cross from one fluid compartment to another at any stage of adult development.

The fact that the epidermis acts as a barrier to many macromolecules suggests that most molting fluid proteins are synthesized by the epidermis itself and are not synthesized elsewhere and transported to the epidermis. This being the case, the presence of five common antigens in both molting fluid and blood suggests that, either (a) the epidermis secretes certain proteins in only one direction, but can secrete other proteins (the five blood antigens) in both directions; or (b) the epidermis secretes the five antigens into the molting fluid and other tissues secrete the same proteins into the blood; (c) the common antigens are secreted by some other tissues into the blood and are transported by the epidermis into the molting fluid. We favor the first suggestion.

These observations lead us to question earlier conclusions that molting fluid contains blood (*e.g.*, Jeuniaux, 1958). It seems likely that, in those cases where molting fluid contains blood, some damage to the delicate new cuticle and epidermis occurred which caused mixing. Indeed, the only *bona fide* cases where blood is to be expected as a normal component of the molting fluid are in insects like *Sialis* in which the larval gills with enclosed blood are trapped in the exuvial space at the time of pupation (Selman, 1960).

It is also worth noting, that, unless great precautions are taken, it is difficult to obtain blood from pharate adult insects without contaminating it with molting fluid. There have been several reports of proteins that appear in the blood only at the time of molting (*e.g.*, McCormick and Scott, 1966). One wonders whether some of these molt-connected blood proteins might be molting fluid proteins that contaminated the blood in the course of collecting blood samples.

As far as the exchange of small molecules between blood and molting fluid is concerned, we have no new data. Wigglesworth (1933) and Jeuniaux (1958)

indicate that dyes of molecular weights up to at least 500 can move from the molting fluid to the blood and Passonneau and Williams (1953) have shown that glycine -C^{14} readily moves from the exuvial space into tissues. Locke (1966) has presented evidence that small molecules are absorbed through small 30 Å pores in the cuticulin of *Calpodes*.

The change in protein composition of molting fluid during adult development requires comment. Recent electron micrographic studies of molting in Apterygota indicate that molting fluid initially consists of granules with inactive enzymes which later become active (Noble-Nesbitt, 1963a, 1963b). The mechanism of this activation is not understood. In the present experiments, there was a great increase in the number of protein bands in molting fluid during the last eight days of adult development, at which time the molting fluid becomes activated. Whether these new bands represent newly-secreted proteins or enzymes, a rearrangement (activation?) of previously secreted proteins, or some cuticular breakdown products is not yet known.

The mechanism for absorption of the molting fluid during the final two days of adult development is unknown. Contrary to the generally held view, the absorption does not take place through the integument as a whole, but through particular regions. In the head and thorax, these regions are principally various flexible membranes at the bases of the appendages. In the abdomen, the principal sites of resorption are pits which represent the points through which tonofibrils make attachment to the old cuticle. In all cases in which it has been studied carefully, the tonofibrils remain attached to the old cuticle until just before ecdysis (Lai-Fook, 1966; Noble-Nesbitt, 1963a, 1963b; Wachter, 1930; Wolfe, 1954). This appears to be true also in developing adult Cecropia. Until the tonofibrils break, the old and new cuticles are closely bound together at the points of attachment. On about day 19, the attachments between the tonofibrils and the pupal cuticle ruptures, and the adult can now move *within* the old pupal cuticle. The cause of this rupturing is not clear, however. As a result of the rupturing, the part of the tonofibrils connecting the two cuticles detaches from the new adult cuticle and leaves the point of attachment on the new cuticle exposed. It is through this exposed surface of adult cuticle—the pit—that molting fluid is absorbed.

The particular ultrastructural features of the tonofibrils that make them suitable sites for resorption requires further study (*cf. e.g.*, Auber, 1963; Bouligand, 1962). Perhaps it is simply that by day 19 most of the cuticle is covered with substances that render it impermeable to molting fluid, whereas the newly exposed cuticular surfaces of the pits are permeable to macromolecules. In any event, although the epidermis on the general surface of the abdomen may be able to absorb molting fluid, in practice, the principal sites of absorption are the pits.

It has been demonstrated previously that, after molting fluid absorption, some molting fluid proteins remain on the surface of the adult cuticle after ecdysis (Wolfe, 1954; Jeuniaux, 1957). However, so far as we are aware no one has demonstrated that macromolecules in the molting fluid get absorbed. The present experiments with rabbit serum proteins demonstrate absorption of foreign proteins from the molting fluid and also demonstrate that these proteins end up *in* rather than *on* the integument. Whether they are in endocuticle, epidermis or both was not determined.

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Summary

1. Molting in insects is always accompanied by the production of a molting fluid which fills the exuvial space between the new and the old cuticle and digests the inner layers of the old cuticle. In *Hyalophora cecropia,* molting fluid is secreted at the outset of adult development and persists until two days before eclosion, whereupon it is absorbed.

2. The present report examines the protein composition of the molting fluid of Cecropia, the origin of the molting fluid proteins, the relation of these proteins to blood proteins and the exchange of macromolecules between the molting fluid and the blood. It also examines the sites of absorption of molting fluid.

3. Disc electrophoresis on acrylamide gels reveals that the molting fluid of Cecropia contains about fifteen protein bands which can be resolved at pH 8.6. Some of these protein bands are detected in the molting fluid at all stages, whereas others appear only at specific times. About ten of the bands are peculiar to molting fluid and are not detected in the blood. About five bands are detectable in both blood and molting fluid, but none of these common bands appears to be a major component of the molting fluid, and only one is a major blood protein. In contrast, the epidermis contains most of the major protein bands found in molting fluid but lacks all but one of the major protein bands present in the blood.

4. Immunological analysis reveals that blood and molting fluid share five antigens. At least four of these common antigens also occur in the epidermis which appears to secrete these antigens into both the molting fluid and the blood.

5. Native and foreign proteins do not penetrate from the exuvial space into the blood or vice versa. Apparently the epidermis and cuticle act as a barrier to the exchange of most macromolecules between the blood and molting fluid. The exuvial space is clearly a separate fluid compartment.

6. In addition the exuvial space itself is compartmentalized and the fluids in the compartments do not admix several days before eclosion.

7. Absorption of molting fluid during the final two days of adult development occurs most readily through particular regions of the integument. In the abdomen the principal sites of absorption are pits which represent the points through which tonofibrils make attachment to the old cuticle. Two days before ecdysis, the attachments between the tonofibrils and the pupal cuticle rupture, exposing the points of attachment on the new cuticle. It is through these exposed surfaces that much of the molting fluid is absorbed. Molting fluid is also absorbed in the head and thorax through various flexible membranes at the bases of the appendages.

LITERATURE CITED

Auber, J., 1963. Ultrastructure de la jonction myo-epidermique des muscles de vol de Dipteres. *J. Microscopie,* 2 : 325–336.

Bouligand, Y., 1962. Les ultrastructures du muscle strié et de ses attaches au squelette chez les cyclops (Crustacés copépodes). *J. Microscopie,* 1 : 377–394.

Chen, P. S., and L. Levenbook, 1966. Studies on the haemolymph proteins of the blowfly, *Phormia regina*. I. Changes in ontogenic patterns. *J. Insect Physiol.*, **12**: 1595–1609.

Davis, B. G., 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. New York Acad. Sci.*, **121**: 404–427.

Grabar, P., and C. A. Williams, 1955. Methode immuno-électrophorétique d'analyse de mélanges de substances antigéniques. *Biochim. Biophys. Acta*, **17**: 67–74.

Jeuniaux, C., 1957. Les enzymes d'origine épidermique au cours du phénomène de la mue chez les insectes. *Actes Société Linnéene, Bordeaux*, **97**: 77–84.

Jeuniaux, C., 1958. Résorption du liquide exuvial chez le ver á soie (*Bombyx mori L.*). *Arch. Int. Physiol. Biochem.*, **66**: 121–122.

Krishnakumaran, A. K., S. J. Berry, H. Oberlander and H. A. Schneiderman, 1967. Nucleic acid synthesis during insect development—II. Control of DNA synthesis in the Cecropia silkworm and other saturniid moths. *J. Insect Physiol.*, **13**: 1–57.

Lai-Fook, J., 1966. The ultrastructure of muscle insertions in insects. *Amer. Zool.*, **6**: 530.

Locke, M., 1966. The structure and formation of the cuticulin layer in the epicuticle of an insect, *Calpodes ethlius* (Lepidoptera, Hesperiidae). *J. Morphol.*, **118**: 461–494.

McCormick, F. W., and A. Scott, 1966. A protein fraction in locust hemolymph associated with the moulting cycle. *Experientia*, **22**: 228–229.

Noble-Nesbitt, J., 1963a. The fully-formed intermoult cuticle and associated structures of *Podura aquatica* (Collembola). *Quart. J. Microscop. Sci.*, **104**: 253–277.

Noble-Nesbitt, J., 1963b. The cuticle and associated structures of *Podura aquatica* at the moult. *Quart. J. Microscop. Sci.*, **104**: 369–391.

Noble-Nesbitt, J., 1967. Aspects of the structure, formation and, function of some insect cuticles. Pages 3–16 in: J. W. Beament and J. E. Treherne, Eds. *Insects and Physiology*, Oliver and Boyd, London.

Ouchterlony, O., 1958. Diffusion-in-gel methods for immunological analysis. *Progr. Allergy*, **5**: 1–78.

Passonneau, J. V., and C. M. Williams, 1953. The moulting fluid of the Cecropia silkworm. *J. Exp. Biol.*, **30**: 545–560.

Patel, N. G., and H. A. Schneiderman, 1969. The effects of perfusion on the synthesis and release of blood proteins by diapausing pupae of the Cecropia silkworm. *J. Insect Physiol.*, **15**: 643–660.

Scheidegger, J. J., 1955. Une micro-methode de l'immuno-electrophoreses. *Int. Arch. Allergy Appl. Immunol.*, **7**: 103–110.

Schneiderman, H. A., and C. M. Williams, 1954. Physiology of insect diapause. IX. The cytochrome oxidase system in relation to the diapause and development of the Cecropia silkworm. *Biol. Bull.*, **118**: 338–351.

Selman, B. J., 1960. On the tissue isolated in some of the larval appendages of *Sialis lutaria* L. at the larval-pupal moult. *J. Insect Physiol.*, **4**: 235–257.

Telfer, W. H., and D. Rutberg, 1960. The effects of blood protein depletion on the growth of the oocytes in the Cecropia moth. *Biol. Bull.*, **118**: 352–366.

Wachter, S., 1930. The moulting of the silkworm and a histological study of the moulting gland. *Ann. Entomol. Soc. Amer.*, **23**: 381–389.

Wigglesworth, V. B., 1933. The physiology of the cuticle and of ecdysis in *Rhodnius prolixus* (Triatomidae, Hemiptera) with special reference to the function of the oenocytes and of the dermal glands. *Quart. J. Microscop. Sci.*, **76**: 269–318.

Wigglesworth, V. B., 1948. The insect cuticle. *Biol. Rev.*, **23**: 408–451.

Wigglesworth, V. B., 1965. *The Principles of Insect Physiology*. Methuen, London, 741 pp.

Wolfe, L. S., 1954. Studies of the development of the imaginal cuticle of *Calliphora erythrocephala*. *Quart. J. Microscop. Sci.*, **95**: 67–78.